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Mammary Gland Tumorigenesis

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FOREWORD

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Note: Copies of publications are attached to this report.

INTRODUCTION

Estrogen exposure represents one of the few clear risk factors for breast cancer in humans. This potential risk is underscored by the finding that both natural and synthetic estrogens are mammary carcinogens in animal models. Despite this clear association between estrogens and breast cancer, the mechanism of tumorigenesis by these agents has not been established. This project will test the hypothesis that estrogens or their metabolites, via covalent binding, are specific disruptors of mitotic microtubules in mammary epithelial cells, and that the resulting interference with mitosis causes aneuploidy. Moreover, the induction of non-random changes in ploidy represents a key step in the malignant transformation of mammary epithelial cells. To test this hypothesis, we will study the metabolism and effects of the mammary carcinogens estradiol (E2), an estrogen, and of 7,12-dimethylbenz[a]anthracene (DMBA), a non-estrogen, in cultures of rat mammary epithelial cells (RMEC). Epithelial cells will be isolated from the mammary glands of female ACI and Sprague-Dawley rats. The former strain is extremely sensitive to estrogens as mammary carcinogens, but quite resistant to the carcinogenic actions of DMBA, whereas the Sprague-Dawley strain exhibits the opposite pattern of sensitivity and resistance. The concentration- and time-dependence of metabolism and the covalent modification of proteins will be determined for each carcinogen in cells from each rat strain, as will the specific covalent modification of tubulin subunits. These chemical endpoints will be compared with the determinations of several cellular changes in response to each of the compounds. Effects on cell proliferation will be assessed by immunohistochemical analysis of the proliferation-specific nuclear antigen PCNA (19A2). Effects on the integrity of microtubules (mitotic spindles) also will be determined immunohistochemically using anti-rat tubulin antibodies. Finally, aneuploidy and chromosomal aberrations will be analyzed by fluorescent *in situ* hybridization (FISH) using reagents and instruments of the Vysis system. These studies of the effects of the compounds on subcellular structure and function constitute a step-wise examination of the proposed mechanism of action for carcinogenic estrogens. Induction of proliferation will be used as a general index of estrogenicity. The effects on microtubules, however, begin to explore the link between covalent modification of tubulin in a target cell for carcinogenicity of these compounds and the disruption of mitosis. If the covalent modification of tubulin, the disruption of microtubules, and the induction of aneuploidy follow the same time- and concentration-dependence then a strong mechanistic link will be forged. This will support future studies on the induction of aneuploidy by estrogens in mammary epithelial cells *in vivo*.

BODY

E2 metabolism by RLM-- The findings reported in the 1998 annual report for this project were confirmed and expanded. Briefly, RLM from both female ACI and SD rats actively carry out the NADPH-dependent oxidation of E2. At E2 concentrations above 1 μM the predominant product is estrone (E1), with lesser amounts of 2-hydroxy-E2 formed. As the substrate concentration is decreased, however, quantitative and qualitative changes occur in these product profiles. The SD microsomes show a progressive shift towards 2-hydroxylation, with diminishing relative amounts of E1 being produced. This fits with 17 β -hydroxysteroid dehydrogenase being a lower affinity enzyme for E2 than are the CYPs involved in hydroxylation reactions. An even more striking change occurs in the ACI RLM. Not only does aromatic hydroxylation become the dominant pathway, but moreover a profound shift from only 2-hydroxylation at high E2 concentration to primarily 4-hydroxylation is seen at nanomolar concentrations of E2. This shift in product profile suggests that a different CYP is present in the ACI rat liver than in the SD preparation. Most CYPs that hydroxylate the A ring of E2 greatly favor attack at the 2-position, rather than at the 4-position. The exception is the recently discovered isozyme CYP 1B1 (1). It is noteworthy that the K_m determined by us for the 4-hydroxylation of E2 in ACI RLM, $0.8 \pm 0.3 \mu\text{M}$, compare favorably with the

value of 0.71 μ M reported by Sutter *et al.* (1) for 4-hydroxylation of E2 by recombinant rat CYP 1B1. We examined the specific CYP content of ACI and SD RLM by performing Western immunoblot analysis.

Analysis for CYP 1A1 and 1A2 was negative for both the ACI and the SD RLM (data not shown). Antibodies against CYP 2B1/2, however, showed that this CYP was expressed in both strains (Fig. 1). Expression levels were noticeably higher in the ACI than in the SD rats. Treatment with s.c. E2 pellets caused some decrease in the levels of CYP 2B detected, but this may not be a significant difference. The highest level of constitutive expression was for CYP 3A1/2 (Fig. 1). E2 treatment did not affect the expression levels. The microsomes from 6 week old rats showed higher 3A levels in the ACI than in the SD rats, but this difference was not apparent in RLM from 12 week old animals. None of these differences correlated with the marked change in product profile between the ACI and the SD rat.

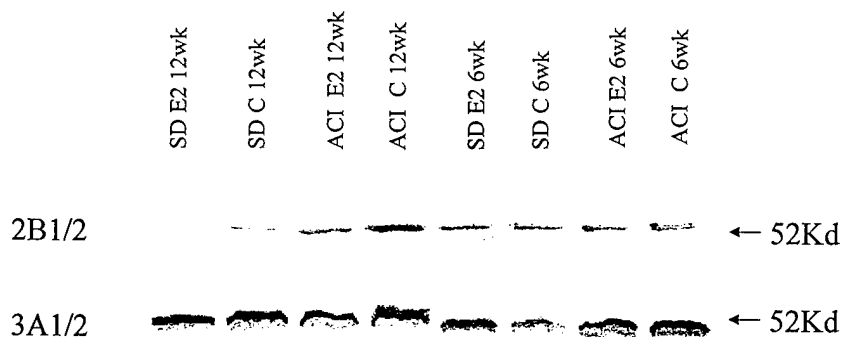


Fig. 1. Western Immunoblot Analysis of RLM from ACI and Sprague-Dawley rats. RLM were prepared from pre- and post-pubertal female rats, both control and those treated with subcutaneous E2 pellets, and analyzed by PAGE followed by immunochemical detection of specific CYPs.

It has become generally accepted that CYP 1B1 is the specific estradiol 4-hydroxylase. It is also assumed that CYP 1B1 is not constitutively expressed in rat liver. In collaboration with Nigel Walker at NIEHS, we analyzed our RLM by Western immunoblotting for CYP 1B1 (Fig. 2). That protein was not detectable in microsomes from either rat strain. The summation of our immunoblotting for CYPs is that there are no significant differences in the expression levels of any of these CYPs between the two strains, and thus no clear enzymological basis for the profound differences in hydroxylation pattern for E2 has been found. We are left with two hypotheses, neither of which will be tested within the current project. The first hypothesis is that a different CYP than those analyzed by our immunoblotting is present in the ACI RLM, and that this as yet unidentified enzyme is the specific high affinity, low capacity 4-hydroxylase responsible for the observed product profile for E2. The second hypothesis is that there is a polymorphism in one of the known CYPs in the ACI rat, and that this polymorphism results in an altered binding orientation for E2 in the active site of the enzyme. This altered binding orientation results in the 4-position of the A ring of the substrate being available for hydroxylation, rather than the 2-position as in the major form of the enzyme. A manuscript is in preparation describing our findings for the metabolism of E2 by RLM.

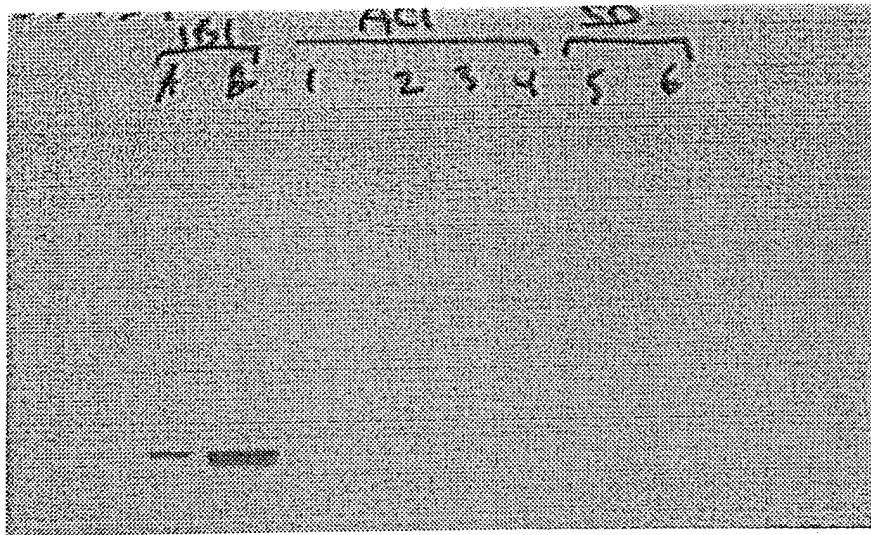


Fig. 2. Western Immunoblot Analysis of RLM from ACI and Sprague-Dawley rats for CYP 1B1. RLM were prepared from post-pubertal female rats and analyzed by PAGE followed by immunochemical detection of CYP 1B1. Lanes A and B contain authentic recombinant CYP 1B1.

RMEC culture-- As noted in the 1998 Annual Report for this project, the preparation of RMEC resulting from collagenase digestion and enrichment by Percoll gradient centrifugation yields myoepithelial cells as the dominant cell type. Although there may be some advantages to having a mix of cells similar to that occurring *in situ*, we have a major concern about this mixed population. First, myoepithelial cells do not appear to be target cells for tumor development. Second, it appears that our initial culture conditions with DMEM/F12 medium and 10% porcine serum select for the preferential growth of the myoepithelial cells at the expense of the luminal and alveolar epithelial cells. We have reviewed the literature and are testing a chemically-defined medium developed for the culture of RMEC by Ip and coworkers (2,3). Our initial studies involve the basal and E2-dependent effects on growth rate of the cells, and on the relative distribution of myoepithelial and luminal epithelial cells. Overall cell proliferation is being measured using a standard MTT tetrazolium salt reduction assay. Myoepithelial cells are detected and counted as actin-positive cells by immunofluorescence, and luminal and alveolar cells are being quantified similarly using peanut agglutinin as a specific probe. We expect that within the month we will have a complete characterization of the distribution of cell types within our RMEC preparations, and more importantly a determination of the effects of E2 on these distributions. Such data are critical for the proper design, performance, and interpretation of the subsequent studies involving E2 and its effects on the rate and possible alterations in cellular replication.

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2. Hahm, H.H., Ip, M.M., Darcy, K., Black, J.D., Shea, W.K., Forczek, S., Yoshimura, M., and Oka, T. (1990) Primary culture of normal rat mammary epithelial cells within a basement membrane matrix. II. Functional differentiation under serum-free conditions. *In Vitro Cell. Dev. Biol.* 26: 803-814.
3. Lee, P.H., Darcy, K.M., Shudo, K., and Ip, M.M. (1995) Interaction of retinoids with steroid and peptide hormones in modulating morphological and functional differentiation in normal rat mammary epithelial cells. *Endocrinology* 136: 1718-1730.

STATEMENT OF WORK

Aim 1.

Task 1. Establish radiometric HPLC assay for estradiol (E2) metabolism	Done
Task 2. Establish radiometric HPLC assay for 7,12-dimethylbenz[a]anthracene (DMBA) metabolism	Done
Task 3. Characterize E2 metabolism in ACI and Sprague-Dawley (SD) rat liver microsomes (RLM)	Done
Task 4. Characterize DMBA metabolism by ACI and SD RLM	Months 25 - 26
Task 5. Optimize culture conditions for ACI and SD rat mammary epithelial cells (RMEC)	Months 20 - 25
Task 6. Characterize E2 metabolism by RMEC from ACI and SD rats	Months 27 - 30
Task 7. Characterize DMBA metabolism by ACI and SD RMEC	Months 27 - 30
Task 8. Characterize covalent modification of ACI and SD RMEC proteins by E2 and DMBA	Months 31 - 35
Task 9. Characterize covalent modification of nuclear DNA in ACI and SD RMEC by E2 and DMBA	Months 31- 33

Aim 2.

Task 1. Optimize immunochemical methods for examination of mitotic microtubule integrity in ACI and SD RMEC	Months 34 - 35
Task 2. Determine concentration dependence of effects of E2 and DMBA on microtubule integrity in ACI and SD RMEC	Months 36 - 37
Task 3. Determine time dependence of effects of E2 and DMBA on microtubule integrity in ACI and SD RMEC	Month 38

Aim 3.

Task 1. Learn and optimize procedures for the detection of aneuploidy in ACI and SD RMEC	Months 39 - 41
Task 2. Determine the concentration dependence of the effects of E2 and DMBA on ploidy in ACI and SD RMEC	Months 42 - 45
Task 3. Determine the time dependence of the effects of E2 and DMBA on ploidy in ACI and SD RMEC	Months 46 - 48

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of 4-hydroxy-E2 as major metabolite of E2 in ACI rat liver
- Determined kinetic constants for 2- and 4-hydroxylation of E2 in ACI and SD rat liver preparations
- Determined that CYP 1B1 is not the E2 4-hydroxylase in the ACI rat liver

PUBLICATIONS

Abstracts

Reed, G.A., Wilson, A.M., and Hayden, J.K.: Estradiol metabolism in rat strains differing in susceptibility to mammary carcinogenesis. *Third International Symposium on Hormonal Carcinogenesis*, 1998.

Wilson, A.M., Padgitt, J.K., and Reed, G.A.: Estradiol metabolism in rat strains differing in susceptibility to mammary carcinogenesis. *Toxicological Sciences* 1-S: 238, 1999.

Manuscripts

Reed, G.A., Wilson, A.M., and Padgitt, J.K.: Estradiol metabolism by rat liver microsomes from strains differing in susceptibility to mammary carcinogenesis. In: *Hormonal Carcinogenesis III* (J.J. Li, S.A. Li, and J.L. Daling, eds.), in press, 1999.

Wilson, A.M. and Reed, G.A.: Predominant 4-hydroxylation of estradiol by constitutive cytochrome P450s in the ACI rat liver. Manuscript in preparation.

FUNDING APPLICATIONS BASED ON THIS WORK

NIH/NCI, "Estrogens, Aneuploidy, and Breast Cancer", Submitted 10/01/98
\$507,061 total direct costs. Not funded. In revision for 11/01/99 submission.

9.

CELLULAR TRANSFORMING ACTIVITY AND GENOTOXICITY OF ESTRADIOL AND ESTRONE, AND THEIR METABOLITES. Tsutsui T*, Tamura Y, Barrett JC. Dept of Pharmacology, The Nippon Dental University, Tokyo 102-8159 and ¹Laboratory of Molecular Carcinogenesis, NIEHS, Research Triangle Park, NC 27709.

To examine a direct involvement of genotoxic effects of estrogens in the initiation of carcinogenesis, the abilities of estradiol (E_2) and estrone (E_1), and their metabolites to induce cellular transformation and genetic effects were examined simultaneously using the Syrian hamster embryo (SHE) cell model. No growth stimulating activities were observed in SHE cells treated with E_2 , E_1 , 2-hydroxyestrone (2-OH- E_1), 4-hydroxyestrone (4-OH- E_1), 2-methoxyestrone (2-Met- E_1), 16 α -hydroxyestrone (16 α -OH- E_1), 2-hydroxyestradiol (2-OH- E_2), 4-hydroxyestradiol (4-OH- E_2), or estriol (E_3). Each compound inhibited SHE cell growth in a dose-dependent manner. Morphological transformation of SHE cells was elicited by each of all compounds, except E_3 . The transforming activities were as follows: 4-OH- E_1 > 2-OH- E_1 > 4-OH- E_2 > 2-OH- E_2 > E_2 \approx E_1 > 2-Met- E_1 \approx 16 α -OH- E_1 > E_3 . Some compounds induced chromosome aberrations in SHE cells with the rank order: 4-OH- E_1 > 2-OH- E_1 \approx 4-OH- E_2 > 2-OH- E_2 > E_1 . Significant increases in the % of aneuploid cells were exhibited by all compounds, except 4-OH- E_1 and E_3 . Somatic mutations at two specific loci were induced by 4-OH- E_1 , 2-Met- E_1 , 2-OH- E_2 , and 4-OH- E_2 . The results indicate that the transforming activities of all estrogens tested correspond to at least one of the genotoxic effects of the compounds, i.e., clastogenicity, aneuploidy, and the ability to cause gene mutations, suggesting the involvement of genotoxicities in the initiation of estrogen carcinogenesis.

10.

EVIDENCE FOR AN INITIATION MECHANISM OF 17 β -ESTRADIOL CARCINOGENESIS. Yu FL*, Wang MY, Bender W. Department of Biomedical Sciences, UIC College of Medicine at Rockford, Rockford, IL 61107.

Recently, we found that 17 β -estradiol (E_2) could be activated by the epoxide-forming oxidant dimethyldioxirane (DMDO) resulting in the inhibition of rat liver nuclear and nucleolar RNA synthesis *in vitro*. Since epoxidation is required for the activation of many chemical carcinogens, we proposed that epoxidation is the mechanism for the initiation of estrogen carcinogenesis (Carcinogenesis 17, 1957-1961, 1996). Initiation requires the binding of a carcinogen to DNA forming DNA adducts. In support of our hypothesis, we found that after activation, [3H]- E_2 was able to bind to DNA, and E_2 -DNA adducts were detected by [^{32}P]-postlabeling (Chemico-Biological Interact. 110,173-187, 1998; Carcinogenesis 19, June issue, 1998). Here, we report that when female ACI rats were given intramammary injections of E_2 and activated E_2 , identical DNA adducts were formed *in vivo*, and that the activated E_2 was at least 25,000-fold more effective in forming DNA adducts in the mammary glands than E_2 . Results from these *in-vitro* and *in-vivo* studies strongly support the initiation role of E_2 in carcinogenesis (Supported by NCI, CA70466).

11.

ESTRADIOL METABOLISM IN RAT STRAINS DIFFERING IN SUSCEPTIBILITY TO MAMMARY CARCINOGENESIS. Reed GA*, Wilson AM, Hayden JK, Department of Pharmacology, Toxicology and Therapeutics, and Kansas Cancer Institute, University of Kansas Medical Center, Kansas City, KS 66160.

Rat strains are known to differ markedly in their susceptibility to estrogen-induced mammary tumors. Specifically, the ACI strain is extremely sensitive to estrogens, whereas the Sprague-Dawley (SD) strain is quite resistant. We have compared the metabolism of estradiol (E_2) by liver microsomes from these two rat strains. Both strains exhibit hydroxysteroid dehydrogenase activity, with estrone (E_1) being the major product at E_2 concentrations above 1 μ M. 2-hydroxyestradiol (2-OH- E_2) also is formed. As the E_2 concentration is decreased, however, hydroxylation becomes a more dominant pathway for both strains. In the SD preparations this still yields E_1 as the major product. ACI liver, however, produces primarily hydroxylated- E_2 at these concentrations. This difference is most apparent at E_2 concentrations below 100 nM. This profound difference in the disposition of E_2 , and that this difference is most pronounced at E_2 concentrations nearing the physiological range, suggest that this difference may contribute to the relative sensitivity of these strains to E_2 carcinogenicity. (Supported by DAMD1 7-97-1-7155 and ES7079).

12.

KINETIC AND BIOCHEMICAL CHARACTERIZATION OF NATURALLY OCCURRING VARIANTS OF THE STEROID 5 α -REDUCTASE TYPE II (SRD5A2) GENE. Makridakis NM, Reichardt JKV*, USC School of Medicine, Los Angeles, CA.

The enzyme 5 α -reductase type II (5 α -RTII), encoded by the SRD5A2 gene in the prostate, is responsible for the conversion of testosterone (T) into its most potent metabolite, dihydrotestosterone (DHT), with NADPH as a cofactor. Here we report the biochemical characterization (using *in-vitro* enzyme assays) of 7 naturally occurring SRD5A2 missense substitutions: 5 missense substitutions show decreased 5 α -RTII activity (with the R227Q mutation displaying a major effect) while the C5R mutation does not affect activity, and the A49T mutation exhibits increased 5 α -RTII activity. In addition, the well-known 5 α -RTII inhibitor finasteride which is utilized for the treatment of benign prostatic hyperplasia and male pattern baldness, can not efficiently inhibit 5 of the 5 α -RTII enzyme variants. Finally, the P30L, A49T, R227Q and F234L mutations seem to define a bipartite binding pocket for T and its competitive inhibitor finasteride.

Enzyme	(T)Km μ M	(NADPH)Km μ M	Vmax nmol/min/mg	(finast.)Ki nM
WT	0.9	8	1.9	23
C5R	0.9	8	1.8	21
P30L	2.1	21	0.5	320
A49T	2.7	7	9.9	270
V89L	0.6	8	1.1	64
T187M	1.1	47	0.8	28
R227Q	4.6	38	0.06	150
F234L	1.6	21	1.4	340

normal mammary tissues. MDA-MB231 cell lines expressed higher AhR levels than the less malignant T47D cell series, irrespective of their estrogen receptor (ER) status. Although TCDD treatment in MDA-MB231 and T47D series have resulted in substantially lowering the AhR levels, the 21T series were less responsive to that effect of TCDD. TCDD treatment resulted in ~5-fold induction of urokinase plasminogen activator (u-PA) in MB231 while dramatically lowering its level in ER-transfected S30 variant of MB231. The plasminogen activator inhibitor-2 (PAI-2) was induced by TCDD in MB231 and ER-negative T47D with the opposite effect in the ER-positive cell lines. While PAI-2 levels in 21T series were inherently high in all three lines and less sensitive to TCDD treatment, the levels of uPA receptor expressed in these cells were proportional to their malignancy in direct correlation to the AhR levels. In conclusion, these data identify the AhR as a key regulator in breast cancer progression, and establish a positive correlation between the AhR expression and components of uPA system. While the AhR activation by TCDD is associated with induction of some components of u-PA system, the lack of this response in the 21T series suggests that the receptor over-expression and its PAH-dependent activation are probably different pathways. The mechanisms underlying these pathways are currently under investigation. (This work was supported by US Army grant DMAD 17-94-J14054.)

1117 EFFECTS OF BROMOETHANE (BE) ON THE ESTROGEN RECEPTOR (ER) AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) LABELING IN HUMAN MCF7 CELLS.

H He, A Yoshida and D Dixon. *National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA.*

BE is a haloethane used primarily as an intermediate in organic synthesis, and in gasoline ethylation. In a 2-year NTP bioassay, BE induced endometrial neoplasms in the uterus of B6C3F1 mice, but decreased the incidence of mammary neoplasms in female F344/N rats. The mechanisms of these effects are unclear; however, we have shown that BE does not have a direct estrogenic effect on the uterus of ovariectomized B6C3F1 mice, but may regulate ER expression. In the present study, we treated estrogen-responsive MCF7 human breast cancer cells with BE at 100, 250, 500, or 1000 mg/L every 24 hours for 3 consecutive days. Cells were harvested on days 2 and 4, and the expression of ER was evaluated using Western blotting and immunohistochemistry. Immunorexpression of PCNA was done to determine the effects of BE treatment on cell proliferation. On day 2, control and treated cells expressed a 66 kDa protein consistent with the ER. This protein was faintly expressed on day 4 by cells treated with 500 or 1000 mg/L of BE. Immunorexpression of ER was observed in control and BE-treated groups on day 2; however, by day 4 cells exposed to 500 or 1000 mg/L did not express ER. The percentage of ER-positive nuclei in cells exposed to 500 or 1000 mg/L of BE was significantly ($p < 0.001$) lower than that of the controls on day 2. On day 4, the PCNA labeling index of cells incubated with 1000 mg/L was significantly ($p < 0.01$) lower than that of controls. These data suggest that continuous exposure of MCF7 cells to BE at high concentrations reduces ER expression and PCNA labeling. These observations may be important in understanding the mechanism of decreased mammary gland neoplasia observed *in vivo*.

1118 NEU ACTIVATION BY ORGANOCHLORINES (OCs) IS CORRELATED TO THEIR ESTROGENIC ACTIONS IN MCF-7 CELLS.

M Hatakeyama, and F Matsumura. *Department of Environmental Toxicology, University of California, Davis, CA, USA.*

The question why some OCs which show very low potency as direct agonists to the estrogen receptor act in an estrogenic manner both *in vitro* and *in vivo* has not been adequately answered yet. In the current study, we examined the effects of selected OCs on activation of Neu, a receptor tyrosine kinase known to play an important role in breast cancer. Following the initial finding made in this laboratory, we studied Neu in a cell free system, in intact cells and 14 day foci formation using human breast cancer MCF-7 cells. The OCs most active in all these tests were alpha-, beta-, and gamma-HCH, o,p'-DDT, o,p'-DDT, 2,2'-dichlorobiphenyl and 2,4,5-T. In all cases the order of potencies during these and less active OCs was essentially identical, indicating the closeness in responses. To ascertain that the long term foci formation is also triggered by the initial activation of Neu kinase, we have tested the inhibitory action of a specific antibody against the ligand binding domain of Neu. The result clearly indicated that the OCs induced foci formation which

is known to be promoted by estrogen, could be specifically suppressed by Neu antibody, but significantly by antibody against EGF receptor. Neu is known to act in a synergistic manner with estrogen in general, and therefore, our current hypothesis is that the activation of these OCs to activate this tyrosine kinase is causally related to their estrogenic actions on cell proliferation activities in estrogen target tissues. (Supported by NIH R01-ES/CA 07284, ES05233 and ES05707.)

1119 ESTRADIOL METABOLISM IN RAT STRAINS DIFFERING IN SUSCEPTIBILITY TO MAMMARY CARCINOGENESIS.

A M Wilson, J K Padgett, and G A Reed. *Department of Pharmacology, Toxicology, and Therapeutics and Kansas Cancer Institute, University of Kansas Medical Center, Kansas City, KS, USA.*

Rat strains are known to differ markedly in their susceptibility to estrogen-induced mammary tumors. Specifically, the ACI strain is extremely sensitive to estrogens, whereas the Sprague-Dawley (SD) strain is quite resistant. We are investigating the basis of this strain difference by comparing the metabolism of estradiol (E2) by liver microsomes from the two strains. Both strains exhibit hydroxysteroid dehydrogenase activity, with estrone (E1) being the major product at E2 concentrations above 1 μ M. 2-Hydroxyestradiol (2-OH-E2) also is formed. As the E2 concentration is decreased, however, cytochrome P450-dependent hydroxylation becomes a more dominant pathway for both strains. At the lower concentrations the SD preparations still yield E1 as the major product. ACI liver, however, produces primarily hydroxylated-E2 at these concentrations. Of particular interest is the production of 4-Hydroxyestradiol (4-OH-E2) as a major product by ACI microsomes. This difference is most apparent at E2 concentrations below 100nM. This profound difference in the disposition of E2, and that this difference is most pronounced at E2 concentrations nearing the physiological range, suggests that this difference may contribute to the relative sensitivity of these strains to E2 carcinogenicity. (Supported by DAMD 17-97-1-7155 and ES-07079.)

1120 INDUCTION OF QUINONE REDUCTASE (QR) AND GLUTATHIONE S-TRANSFERASE (GST) BY GREEN TEA EPIGALLOCATECHIN GALLATE AND QUERCETIN IN MCF-7 HUMAN BREAST CANCER CELLS.

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The polyphenolic compounds quercetin and green tea epigallocatechin gallate (EGCG) are flavonoids which have potent chemopreventive activity in a variety of animal models of cancer. One proposed mechanism for the inhibition of chemical carcinogenesis by these agents is induction of phase-II detoxification enzymes. In this investigation, the effects of quercetin, commercial green tea extract, and EGCG on GST and QR activities were evaluated in MCF-7 human breast cancer cells. Following 24 hr treatment with flavonoids, a maximum 2-fold increase in GST and QR enzyme activities were demonstrated in this cell model. Quercetin caused a significant increase in QR protein expression, as measured by western blot analysis. These results demonstrate that major dietary polyphenols, such as quercetin, green tea EGCG and whole green tea extract are capable of inducing phase-II enzymes in MCF-7 cells. The induction of these enzymes *in vivo* would result in the elimination of reactive metabolites of chemical carcinogens. This suggests a mechanism for chemoprevention of mammary cancer by flavonoids.

1121 PSP94 EXPRESSION IN PROSTATE CANCER PERSISTS AFTER ANDROGEN DEPRIVATION THERAPY.

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PSP94 is one of the three major proteins secreted by prostate gland. In contrast to PSA and PAP, PSP94 was found to be androgen-independent *in vitro*. This prompted us to study the effect of hormonal therapy on PSP94 expression in prostate cancer. 87 radical prostatectomies on untreated patients (Group 1) and 74 from patients with neoadjuvant hormonal therapy (Group 2) were studied. Formalin-fixed, paraffin-embedded tissues were stained by ABC technique using a primary rabbit antibody against human PSP94 purified from semen. Tumor foci on each section were identified and were graded according to the scheme of Gleason. The intensity of the staining was graded on a scale of 0 to 2+, and the extent of the staining was classified as

Estradiol Metabolism by Rat Liver Microsomes from Strains Differing in Susceptibility to Mammary Carcinogenesis

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Summary

Rat strains are known to differ markedly in their susceptibility to estrogen-induced mammary tumors. Specifically, the ACI strain is extremely sensitive to estrogens, whereas the Sprague-Dawley (SD) strain is relatively resistant. We have compared the metabolism of estradiol (E2) by liver microsomes from these two rat strains. Both strains exhibit hydroxysteroid dehydrogenase activity, with estrone (E1) being the major product at E2 concentrations above 1 μ M. Some A-ring hydroxylation also is seen. As the E2 concentration is decreased, however, hydroxylation becomes a more dominant pathway for both strains. In the SD preparations this still yields E1 as the major product. ACI liver, however, produces primarily hydroxylated-E2 at these concentrations. This difference is most apparent at E2 concentrations below 100 nM. This profound difference in the disposition of E2, and that this difference is most pronounced at E2 concentrations nearing the physiological range, suggest that this difference may contribute to the relative sensitivity of these strains to E2 carcinogenicity.

Introduction

The identification of carcinogenic risks may be derived from two complementary approaches: the first is epidemiological analysis of human populations, while the second is by extrapolation from controlled exposure studies with experimental animals. The association between estrogens and breast cancer has been supported by ample data from both approaches. Despite the clear association between estrogens and breast cancer, the mechanisms involved in this effect are not clear.

A pronounced strain difference in sensitivity to mammary carcinogenesis by estrogens provides an opportunity to identify key mechanistic features of the process. The ACI rat is sensitive to estrogens as mammary carcinogens (1-4). Sprague-Dawley rats, in contrast, are relatively resistant to estrogen carcinogenicity (2,5). By comparing estrogen metabolism and resultant effects in the two strains it not

only is possible to look for differences in metabolism and responses, but also to try and correlate these responses with susceptibility to carcinogenesis. The examination of estradiol (E2) metabolism by liver microsomal preparations from these two strains represents the first step in these comparative studies.

Materials and Methods

Female Sprague-Dawley and ACI rats, 6 to 7 weeks old, were purchased from Harlan Laboratories. The livers were homogenized and microsomes prepared by differential centrifugation. Microsomal pellets were resuspended in phosphate-MgCl₂ buffer, pH 7.4, and stored at -80° C.

[³H]-E2 was incubated with RLM (1 mg protein/ml) in 50mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂, 5 mM glucose-6-phosphate, and 1 mM ascorbate at 37° C. The NADPH generating system, where noted, included 1 U/ml glucose-6-phosphate dehydrogenase and was initiated by the addition of 1 mM NADP. Reactions with NADP or NAD as cofactors were initiated by the addition of these compounds, again to a final concentration of 1 mM. Incubations were continued for 10 to 60 min, as indicated, depending on substrate concentration. The reaction was stopped by the rapid extraction with 2 x 3 vol of ethyl acetate. The combined extracts were evaporated under vacuum, and the residue dissolved in the initial acetonitrile-methanol-water-acetic acid mix of the HPLC elution.

Separation of metabolites was performed on a Supelcosil C18 column (5 μ , 4.6 mm x 25 cm) at 30° C using a gradient of acetonitrile, methanol, water, and 0.1% acetic acid (6). Unlabeled metabolite standards were detected by monitoring absorbance at 280 nm, whereas labeled metabolites were detected by a Packard Flo-One Beta detector with Ultima Flo M liquid scintillant.

Results

Liver microsomal preparations from female ACI and SD rats both catalyze extensive NADPH-dependent metabolism of E2. Oxidation of E2 to E1 and aromatic hydroxylation of E2 were the only products observed with these preparations. Maximal rates of E1 formation exceed 300 pmol mg⁻¹ min⁻¹

¹ in both strains, whereas maximal rates of aromatic hydroxylation are greater than 6 pmol mg⁻¹ min⁻¹ in SD microsomes and 30 pmol mg⁻¹ min⁻¹ in ACI liver microsomes. Extensive metabolism was observed not only at the commonly used micromolar concentrations of E2 (7-10), but at substrate concentrations as low as 3 nM.

Although the metabolite profiles derived from the two strains are nearly identical at high substrate concentrations, a pronounced divergence emerges as the E2 concentration drops below 1 µM (Fig. 1). For both strains, aromatic hydroxylation becomes more pronounced as the E2 concentration is decreased, but this shift is far more extensive in the ACI rat. As shown, at 100 nM E2 aromatic hydroxylation to yield catechol estrogens becomes the dominant pathway for E2 oxidation, comprising 73% of total metabolism. In the SD rat preparation, however, the fraction of metabolism resulting from aromatic hydroxylation only reaches 36% of the total. An additional 16% of the metabolites produced by the SD rat are catechol E1 derivatives, which have undergone both 17β-dehydrogenation but also aromatic hydroxylation. Even combining the E1 and E2 catechol metabolites still only comprises 52% of total metabolism, as compared to the 73% catechols formed by the ACI liver microsomes under these same conditions.

The formation of hydroxylated products is assumed to be a cytochrome P450-dependent reaction, and thus NADPH-dependent, whereas the conversion to E1 is a dehydrogenation which may utilize either NAD or NADP as a cofactor. This is borne out by the cofactor specificity demonstrated in Table 1. With the active glucose-6-phosphate dehydrogenase generating system maintaining most NADPH in the reduced form, fully 75% of E2 metabolism is by hydroxylation, rather than dehydrogenation. The use of NADP, without the generating system for reduction of the cofactor, results in a 3.5-fold increase in the rate of dehydrogenation to E1, and a 70% decrease in aromatic hydroxylation. The residual hydroxylation presumably results from reduction of NADP via the 17β-hydroxysteroid dehydrogenase reaction. Finally, when NAD is added as the cofactor, which is fully active as a 17β-hydroxysteroid dehydrogenase cofactor but inactive as a cytochrome P450 cofactor in either its

oxidized or reduced form, no hydroxylation is observed and 100% of the E2 is converted to E1. These cofactor studies clearly support the assignment of E1 formation to 17 β -hydroxysteroid dehydrogenase and the aromatic hydroxylation as a cytochrome P450-dependent reaction.

Conclusions

1. Rat liver microsomes efficiently oxidize E2 at concentrations as low as 3 nM.
2. 17 β -Hydroxysteroid dehydrogenase is a relatively low affinity but high capacity system for E2 oxidation in RLM. Cytochrome P450-dependent aromatic hydroxylation of E2 is a relatively high affinity but low capacity system for E2 oxidation in RLM.
3. RLM from the female ACI rat differ from those from other rat strains in that aromatic hydroxylation of E2 is more dominant than it is in the SD rat.

This difference in aromatic hydroxylation of E2 between the ACI rat and the Sprague-Dawley rat will be investigated further and findings will be considered relative to the pronounced difference between these strains in their sensitivity to E2 carcinogenicity.

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Table 1. Cofactor Effect on E2 Oxidation by ACI Rat Liver Microsomes

<u>Cofactor</u>	<u>Aromatic Hydroxylation</u>	<u>Dehydrogenation</u>
NADPH generating system	$25.8 \pm 1.1 \text{ pmol mg}^{-1} \text{ min}^{-1}$	$8.7 \pm 2.2 \text{ pmol mg}^{-1} \text{ min}^{-1}$
NADP	6.3 ± 0.8	20.5 ± 0.5
NAD	0	50 ± 0

1 μM E2 was incubated for 20 minutes with 1 mg/ml microsomal protein and either the NADPH generating system or with 1 mM NADP or NAD. Extraction and analysis were as described in Materials and Methods. Results are means \pm standard deviation from triplicate incubations.

Figure 1. Product Distribution from E2 Oxidation by ACI and SD Rat Liver Microsomes:**Effect of Substrate Concentration**

Indicated concentrations of [^3H]-E2 were incubated with 1 mg/ml microsomal protein with an NADPH generating system for 20 minutes. Extraction and analysis were as described. All data represent mean \pm standard deviation from triplicate incubations. A, ACI rat, 100 nM E2; B, ACI rat, 30 μM E2; C, SD rat, 100 nM E2; D, SD rat, 30 μM E2.